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## **A Natural Killer Cell Receptor Specific for a Major Histocompatibility Complex Class I Molecule**

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### **Summary**

Target cell expression of major histocompatibility complex (MHC) class I molecules correlates with resistance to lysis by natural killer (NK) cells. Prior functional studies of the murine NK cell surface molecule, Ly-49, suggested its role in downregulating NK cell cytotoxicity by specifically interacting with target cell H-2D<sup>d</sup> molecules. In support of this hypothesis, we now demonstrate a physical interaction between H-2D<sup>d</sup> and Ly-49 in both qualitative and quantitative cell-cell binding assays employing a stable transfected Chinese hamster ovary (CHO) cell line expressing Ly-49 and MHC class I transfected target cells. Binding occurred only when CHO cells expressed Ly-49 at high levels and targets expressed H-2D<sup>d</sup> by transfection. Monoclonal antibody blocking experiments confirmed this interaction. These studies indicate that the specificity of natural killing is influenced by NK cell receptors that engage target cell MHC class I molecules.

NK cells spontaneously lyse certain transformed or virally infected cells but do not kill normal cells (1, 2). Although the molecular basis for NK cell specificity is poorly understood, target cell susceptibility to spontaneous cytotoxicity is inversely proportional to their level of expression of certain MHC class I molecules (3–5). Two explanations (6) have been proposed. MHC class I molecules may “mask” or interfere with recognition of putative target cell ligands by activation receptors on NK cells. Alternatively, target cell MHC class I molecules may directly engage NK cell receptors leading to inhibitory events in NK cells. Our previous functional studies (7) of the murine Ly-49 molecule have supported the latter hypothesis.

Ly-49 is a type II integral membrane protein (8, 9), with an external C-type lectin domain (9), expressed on a distinct subset of murine NK cells (10) as a disulfide-linked homodimer of 44-kD subunits (9). Functional studies with IL-2-activated NK cells demonstrated that Ly-49<sup>+</sup> NK cells generally manifested a lytic capacity equivalent to Ly-49<sup>−</sup> NK cells (7). However, Ly-49<sup>+</sup> NK cells were unable to lyse targets of H-2<sup>k</sup> or H-2<sup>d</sup> haplotype despite efficient lysis of these targets by Ly-49<sup>−</sup> NK cells, demonstrating that Ly-49 expression significantly influences NK cell target specificity. Transfection of a susceptible target cell line with cDNA encoding H-2D<sup>d</sup> rendered it resistant to lysis by Ly-49<sup>+</sup> NK cells. Ly-49<sup>+</sup> NK cells were unable to lyse the D<sup>d</sup> transfect-

ant by other stimuli, including Ab-dependent cellular cytotoxicity (7, 11). Gene transferred resistance was abrogated by mAbs against either Ly-49 or the  $\alpha 1/\alpha 2$  domain of D<sup>d</sup>. These data were compatible with our hypothesis that Ly-49 is an inhibitory receptor that specifically recognizes D<sup>d</sup> molecules on targets.

Our interpretation of these functional studies predicts that Ly-49 directly interacts with H-2D<sup>d</sup>. Alternatively, the results may be explained by a NK cell surface molecule that is coexpressed with Ly-49 and that is indirectly influenced by the anti-Ly-49 mAb. Moreover, reversal of functional inhibition by mAbs could be due to other effects such as direct anti-Ly-49 triggering or anti-H-2D<sup>d</sup> dissociation of another effector cell ligand (12). A final possibility involves the “masking model” in which H-2D<sup>d</sup> masks another target molecule specifically recognized by Ly-49 (13). To distinguish between these possibilities, we sought to unequivocally establish the ligand for Ly-49. We have now overexpressed Ly-49 on Chinese hamster ovary (CHO) cells by transfection and DNA amplification. CHO cells bind target cells only when the cells express high levels of Ly-49 and H-2D<sup>d</sup>, respectively, by transfection. This interaction is specifically blocked by appropriate mAbs. Thus, this study demonstrates that Ly-49 is a receptor for a MHC class I molecule, further substantiating a role for target cell MHC class I molecules in innate immunity in addition to acquired (T cell-mediated) immunity.

## Materials and Methods

**Cell Culture.** All cell lines were maintained in RPMI 1640 (Mediatech Inc., Washington, D.C.) supplemented with 10% FCS (HyClone Labs., Logan, UT) 100 U/ml penicillin K, 0.1 mg/ml streptomycin sulphate, and 0.3 mg/ml L-glutamine (GIBCO BRL, Gaithersburg, MD). CHO cells, deficient in the dihydrofolate reductase gene (*dhfr*<sup>-</sup>), were grown in Ham F-12 media with the above supplements. The C1498 transfectants, expressing D<sup>d</sup>, K<sup>d</sup>, or L<sup>d</sup>, were described previously (7) and were maintained in media containing 0.5 mg/ml G418 (GIBCO BRL).

**mAbs.** The mAbs A1 (anti-Ly-49) and PK136 (anti-NK1.1) have been previously described (14, 15). The anti-MHC mAbs were 34-5-8S (anti- $\alpha$ 1/ $\alpha$ 2 domains of D<sup>d</sup>, 34-2-12 (anti- $\alpha$ 3 domain of D<sup>d</sup>), and B22.249 (anti-D<sup>b</sup>) (16, 17). All anti-MHC mAbs bind to C1498-D<sup>d</sup> target cells at the same density by flow cytometry (data not shown).

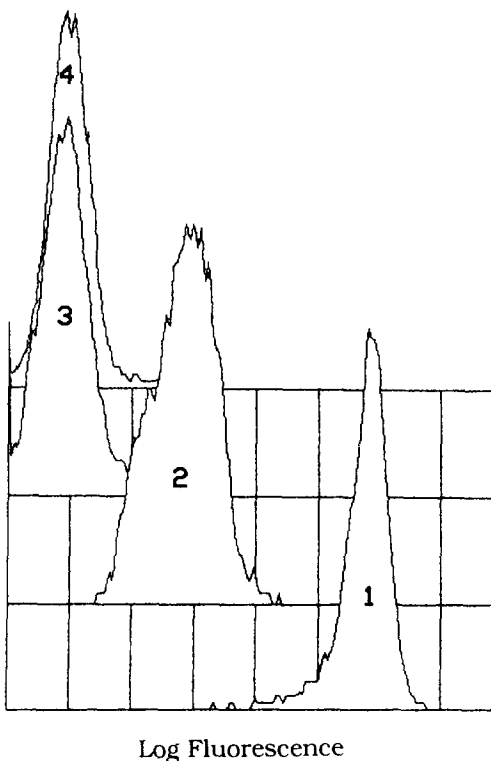
**Production and Amplification of Ly-49<sup>+</sup> CHO Cells.** The HindIII fragment of pA1.3 (9) was subcloned into the HindIII site of pH $\beta$ APr-1 neo (18). Constructs containing the Ly-49 cDNA in the sense and inverse (Inv) orientation with respect to the promoter were identified and cloned. The EcoRI-PvuII fragment of pSV2-dhfr (19) containing the dhfr cDNA was subcloned into the EcoRI site of both Ly-49 expression constructs. The resulting plasmids contained the Ly-49 cDNA (in either the sense or inverted orientations) under the control of the human  $\beta$ -actin promoter and the dhfr cDNA under the control of the SV40 early promoter. The plasmids were linearized by EcoRI digestion and individually electroporated (Gene Pulser; Bio-Rad Laboratories, Hercules, CA) into *dhfr*<sup>-</sup> CHO cells. Resistant cells were initially selected and cloned in 1.5 mg/ml G418. Clones were then transferred to  $\alpha$ -MEM media (ICN/Flow, Costa Mesa, CA) with 10% dialyzed FCS. Cells transfected with the Ly-49 sense construct, that grew in both selection medias and expressed Ly-49 on their surface by FACS<sup>®</sup> analysis (Becton Dickinson & Co., Mountain View, CA), were subjected to three rounds of amplification with successively higher concentrations of methotrexate (MTX; Sigma Chemical Co., St. Louis, MO) and maintained at 1.28  $\mu$ M MTX to establish Ly-49<sup>High</sup> CHO cells. In parallel cultures, CHO cells transfected with the inverse oriented construct were subjected to the same selection and three rounds of MTX amplification but clones were randomly picked. The Ly-49<sup>Inv</sup> CHO cells were also maintained in 1.28  $\mu$ M MTX. Ly-49<sup>Low</sup> CHO cells were isolated from cells transfected with the sense construct after a single cycle of MTX amplification and were maintained in 20 nM MTX.

**Rosette Assay.** Ly-49<sup>High</sup> CHO or Ly-49<sup>Inv</sup> CHO cells ( $3 \times 10^4$ ) were grown overnight in 24-well tissue culture plates and washed twice with wash buffer (HBSS with 3% FCS). The targets were washed twice with cold wash buffer and diluted to  $6 \times 10^5$  cells/ml; 500  $\lambda$  aliquots were added to each well and incubated for 45 min in a 37°C, 5% CO<sub>2</sub> incubator. Wells were washed twice with wash buffer and photographed with a phase contrast inverted microscope.

**Quantitative Binding Assay.** This assay uses centrifugation of inverted 96-well plates to remove unbound labeled target cells from CHO cell monolayers, permitting reproducibility and objectivity (modified from 20). In this assay, CHO cells ( $6 \times 10^3$ ) were grown overnight in a 96-well tissue culture plate. Target cells were washed twice with RPMI 1640 (without FCS). The fluorescent dye, BCECF-AM (2',7'-bis-[2-carboxyethyl]-5-[and-6]-carboxyfluorescein, acetoxymethyl ester; Molecular Probes, Inc., Eugene, OR), was solubilized in DMSO and diluted to 50  $\mu$ g/ml in RPMI 1640. The target cells ( $4 \times 10^6$ ) were resuspended in 1 ml of diluted BCECF-AM and incubated for 2 h in a 37°C, 5%

CO<sub>2</sub> incubator. Target cells were then washed twice with cold wash buffer and diluted to  $1.6 \times 10^6$  cells/ml. 50  $\lambda$  aliquots were added to washed CHO cell monolayers and returned to the CO<sub>2</sub> incubator for 45 min. Wells were filled to a bulging meniscus with wash buffer, sealed with waterproof tape, inverted, and centrifuged in the upside down position at 300 g for 4 min. While still inverted, the sealant tape was removed, the plate drained, blotted, and reverted to the upright orientation. The remaining cells were lysed with 100  $\lambda$  of lysis buffer (25 mM Tris, 0.1% SDS). 100  $\lambda$  aliquots from each well were transferred into a disposable cuvette filled with 4 ml of lysis buffer. Relative fluorescence (RF) (compared to buffer alone) was determined with a spectrofluorimeter (Fluorolog 2; Spex, Inc., Edison, NJ) at 502 nm excitation and 530 nm emission. Percent specific binding was calculated with the following formula:  $(RF_{\text{sample}} - RF_{\text{dhfr-CHO}}) / (RF_{\text{max}} - RF_{\text{dhfr-CHO}})$ .  $RF_{\text{sample}}$  is the RF of the experimental sample whereas  $RF_{\text{dhfr-CHO}}$  is the background fluorescence remaining in wells with untransfected *dhfr*<sup>-</sup> CHO cells (always <8% of  $RF_{\text{max}}$ ) and  $RF_{\text{max}}$  is the total fluorescence added to each well as measured by fluorescence of unwashed wells with labeled targets and *dhfr*<sup>-</sup> CHO cells. The mean of duplicate wells for each point was used in the calculation. In experiments that used mAbs, saturating concentrations (1:200–1:500) of ascites preparations were added to target cells or to CHO cell monolayers 30 min before the assay.

**FACS<sup>®</sup> Analysis.** A FACScan<sup>®</sup> with the Consort 32 software program (Becton Dickinson & Co.) was used to analyze CHO cells for Ly-49 expression with mAb A1 (anti-Ly-49) as described previously (7).



**Figure 1.** Expression of Ly-49 on CHO cells. Ly-49<sup>High</sup> (histogram 1), Ly-49<sup>Low</sup> (2), Ly-49<sup>Inv</sup> (3), and parental *dhfr*-deficient CHO cells (4) were indirectly stained with anti-Ly-49 mAb and FITC-conjugated goat anti-mouse Ig, and analyzed by flow cytometry as described (7). Background staining of Ly-49<sup>High</sup> and Ly-49<sup>Low</sup> cells (either unstained or with second-step reagent alone, data not shown) overlap histograms 3 and 4.

## Results

**Ly-49<sup>High</sup> CHO Cells Rosette H-2D<sup>d</sup> Targets.** To determine a specific physical interaction between Ly-49 and H-2D<sup>d</sup>, our experimental approach employed binding assays between transfected cells expressing high levels of Ly-49 and transfected target cells expressing various MHC class I molecules. Similar systems successfully established the physical interaction between CD4 and CD8 and their respective MHC ligands (21, 22). The *dhfr*<sup>-</sup> CHO cells were transfected with an expression plasmid containing the Ly-49 cDNA and *dhfr*. Ly-49 expression was amplified by exposing positive clones to successively higher concentrations of MTX. Two cell lines, Ly-49<sup>High</sup> and Ly-49<sup>Low</sup> CHO cells were developed. By flow cytometry, Ly-49<sup>High</sup> CHO cells expressed Ly-49 at 30–40 times the level on Ly-49<sup>Low</sup> CHO cells (Fig. 1) which in turn was comparable to Ly-49<sup>+</sup> NK cells constitutively expressing Ly-49 (data not shown). Immunoprecipitation analysis with mAb A1 (anti-Ly-49) showed that Ly-49 was appropriately expressed on Ly-49<sup>+</sup> CHO cells as a disulfide-linked homodimer with the expected molecular mass (data not shown). In parallel cultures, a control cell line, Ly-49<sup>Inv</sup> CHO, was produced by transfection with a plasmid containing an inverted Ly-49 cDNA and subjected to the same three rounds of MTX amplification as Ly-49<sup>High</sup> CHO cells. Parental and Ly-49<sup>Inv</sup> CHO cells did not express Ly-49 (Fig. 1).

Ly-49<sup>High</sup> CHO cells were qualitatively able to rosette transfected cells expressing H-2D<sup>d</sup> (C1498-D<sup>d</sup>), whereas untransfected C1498 and C1498-K<sup>d</sup> cells did not bind (Fig. 2). Binding of C1498-D<sup>d</sup> cells was specific for Ly-49<sup>High</sup> CHO

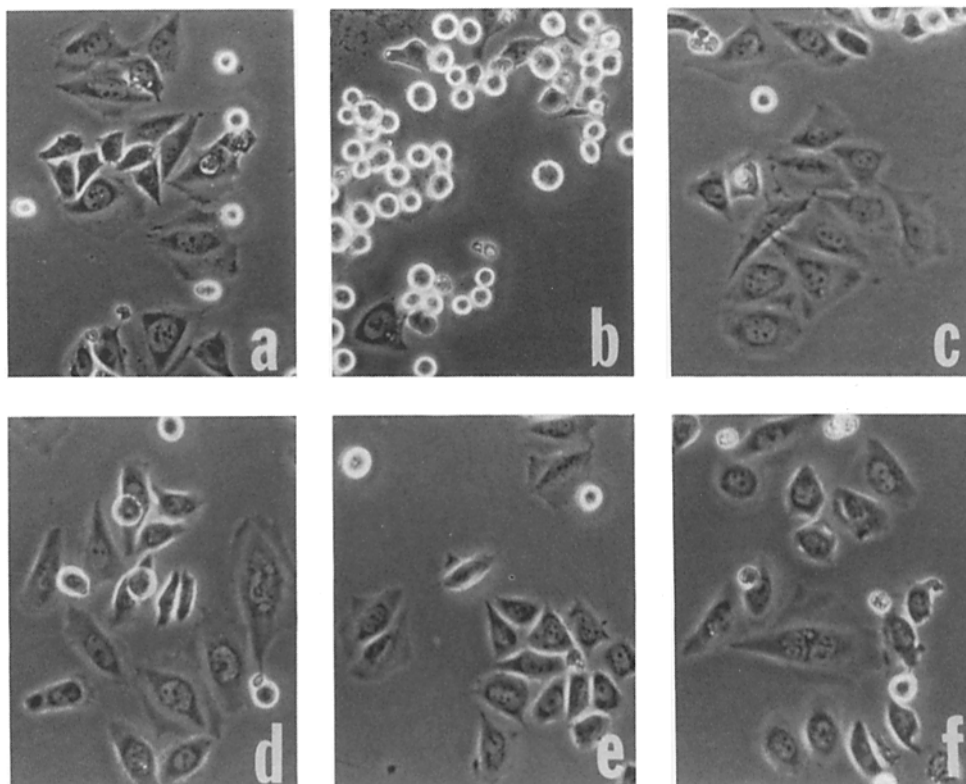
cells because they did not bind Ly-49<sup>Inv</sup> CHO cells. Thus, rosette formation between these transfected cells appeared to be dependent on a specific interaction between Ly-49 and H-2D<sup>d</sup>.

**Ly-49 Specifically Recognizes H-2D<sup>d</sup>.** To eliminate any subjective bias in our rosette analysis, a quantitative assay was developed in which the only detectable binding was between Ly-49<sup>High</sup> CHO cells and C1498-D<sup>d</sup> cells (Fig. 3 A). Untransfected C1498, C1498-K<sup>d</sup>, and C1498-L<sup>d</sup> did not adhere to Ly-49<sup>High</sup> CHO cells. The Ly-49<sup>Inv</sup> CHO cells did not bind any targets. A high level of Ly-49 expression was required for binding because C1498-D<sup>d</sup> cells did not adhere to Ly-49<sup>Low</sup> CHO cells (Fig. 3 B). Thus, in rosette and quantitative assays, adhesion between transfected CHO and C1498 cells demonstrated an interaction between Ly-49 and H-2D<sup>d</sup>.

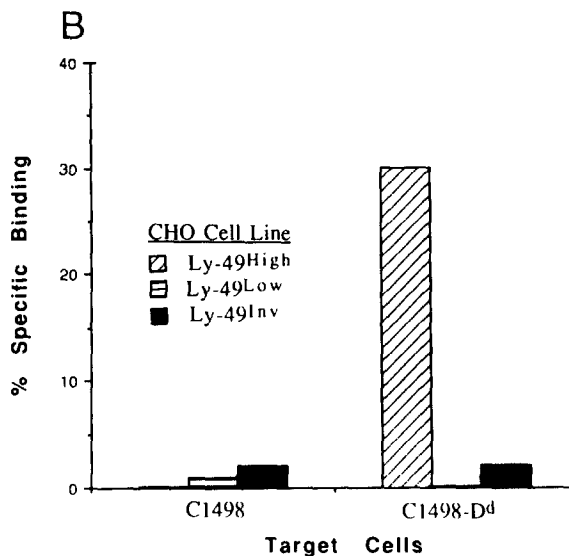
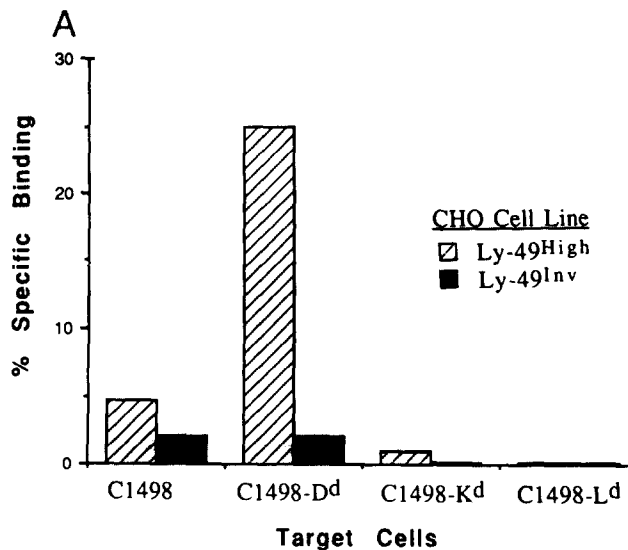
The specificity of this interaction was further confirmed by mAb blocking studies. Preincubation of Ly-49<sup>High</sup> CHO cells with mAb A1 (anti-Ly-49) prevented binding to C1498-D<sup>d</sup> cells, whereas an isotype control mAb had no effect (Fig. 4). Incubation of C1498-D<sup>d</sup> cells with a mAb specific for  $\alpha 1/\alpha 2$  domains of D<sup>d</sup> also blocked binding. mAbs directed against D<sup>b</sup> or the  $\alpha 3$  domain of D<sup>d</sup> had no effect despite equivalent binding of all mAbs to C1498-D<sup>d</sup> cells by FACS<sup>®</sup> analysis (data not shown). Similar analysis with the rosette assay confirmed these results (data not shown).

## Discussion

In this report, we describe a physical interaction between Ly-49 and H-2D<sup>d</sup> in binding assays in which target cells



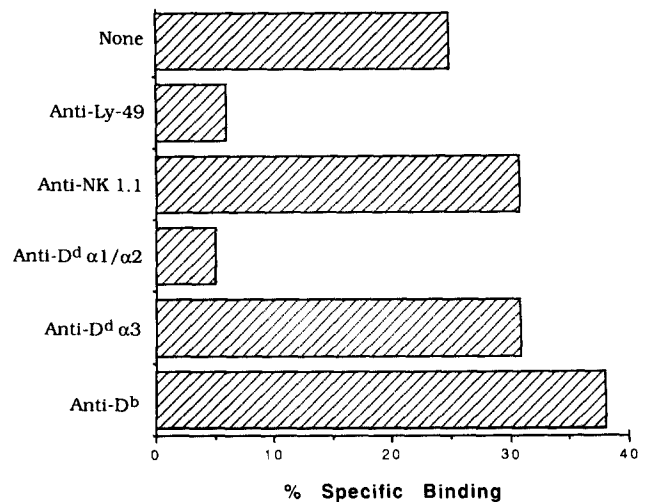
**Figure 2.** Ly-49<sup>High</sup> CHO cells rosette H-2D<sup>d</sup>-expressing target cells. Parental C1498 (a and d) or C1498 cells transfected with cDNAs encoding D<sup>d</sup> (b and e) or K<sup>d</sup> (c and f) were assayed for ability to rosette with Ly-49<sup>High</sup> (a–c) or Ly-49<sup>Inv</sup> CHO cells (d–f). The CHO cells appear as large polygonal cells whereas target cells are small, round, and refractile.  $\times 50$ .



**Figure 3.** Quantitative binding of target cells expressing H-2D<sup>d</sup> to Ly-49<sup>High</sup> CHO cells. (A) Ly-49<sup>High</sup>, or Ly-49<sup>Inv</sup> CHO cells were tested for ability to bind parental C1498 target cells, or C1498 cells transfected with cDNAs encoding H-2D<sup>d</sup>, K<sup>d</sup>, or L<sup>d</sup> in a quantitative fluorescence binding assay. (B) Binding depends on level of Ly-49 expression on CHO cells. Ly-49<sup>High</sup>, Ly-49<sup>Low</sup>, and Ly-49<sup>Inv</sup> CHO cells were assayed for binding parental C1498 cells or C1498-D<sup>d</sup> cells.

adhere to CHO cells only when the target expressed H-2D<sup>d</sup> and the CHO cell expresses Ly-49 at high levels. The specificity of this interaction was therefore established in a system in which adherent cells differed from nonadherent cells only by transfection and expression of the relevant cDNAs. mAb blocking experiments confirmed this specificity. Thus, Ly-49 specifically engages H-2D<sup>d</sup> molecules.

While this manuscript was being prepared, complementary results were reported by Kane (23) who showed that



**Figure 4.** mAbs specific for Ly-49 or α1/α2 domains of H-2D<sup>d</sup> block binding. Quantitative binding between Ly-49<sup>High</sup> CHO cells and C1498-D<sup>d</sup> cells was assayed in the absence or presence of the indicated mAbs. Ly-49<sup>Inv</sup> CHO cells failed to bind target cells (Fig. 3 and data not shown).

the ability of tumor cells, such as EL-4, to bind immobilized immunopurified H-2D<sup>d</sup> could be correlated with Ly-49 expression. Binding of EL-4 was also detected to H-2K<sup>b</sup> and H-2D<sup>k</sup>. Previous NK cell functional results predicted that Ly-49 should bind to H-2D<sup>d</sup> and an H-2k-MHC class I product, consistent with H-2D<sup>k</sup>, as reported (7, 23). However, Ly-49 belongs to a family of highly related molecules (24, 25) encoded by crosshybridizing transcripts of similar size. It thus remained possible that EL-4 cells could express these related molecules, each binding different MHC class I molecules. Although anti-Ly-49 mAb specifically blocked EL-4 binding to immunopurified H-2D<sup>d</sup> and H-2D<sup>k</sup>, the mAb could recognize Ly-49-related molecules. Another possible interpretation of the EL-4 experiments is that the mAb could sterically hinder binding of an unrelated molecule, coexpressed and physically associated with Ly-49, that actually mediates binding to MHC class I molecules. Our results eliminate these possible alternative interpretations because binding only occurred when paired cell lines express Ly-49 and H-2D<sup>d</sup>, respectively, by transfection.

Our current results also differ from the EL-4 binding studies in the anti-H-2D<sup>d</sup> mAb blocking experiments and in binding to H-2K<sup>b</sup> (23). In EL-4 binding, the H-2D<sup>d</sup>-α3 domain appeared to be nearly as important as the α1/α2 domain. In contrast, functional studies demonstrated that the same anti-H-2D<sup>d</sup>-α3 domain mAb had no effect, suggesting that the α3 domain is not involved (7). Other studies (26, 27) have also demonstrated that specific amino acids in the α1 helix can regulate MHC class I-associated target cell susceptibility to NK cells. A single substitution in HLA-A2 (His-74 to Asp-74) converted a nonprotective phenotype into one that was protective (26). A possible explanation for this discrepancy is that other cell surface molecules on EL-4 cells may contribute to its binding to immunopurified MHC class

I molecules. Similarly, EL-4 binding to H-2K<sup>b</sup> differed from its binding to H-2D<sup>d</sup> and H-2D<sup>k</sup> because the anti-Ly-49 mAb did not block binding of EL-4 to H-2K<sup>b</sup> (23). Indeed, there was no prior evidence suggesting that H-2K<sup>b</sup>, expressed by several sensitive targets, had any effect on cytotoxicity by Ly-49<sup>+</sup> effector cells (7). Moreover, in our current experiments, the various C1498 targets express H-2K<sup>b</sup> normally and at the same levels (7) but do not bind Ly-49<sup>High</sup> CHO cells. Thus, binding of Ly-49<sup>+</sup> EL-4 cells to H-2K<sup>b</sup> is not due to Ly-49 and may be due to other molecules expressed by EL-4.

Our current results suggest that binding of Ly-49 to H-2D<sup>d</sup> primarily involves the peptide binding domains of MHC class I molecules in a manner similar to interaction of MHC class I-restricted TCRs rather than CD8 molecules (28, 29). The possible binding of Ly-49 to the H-2D<sup>d</sup>- $\alpha 1/\alpha 2$  domain suggests that bound peptides may alter MHC class I-associated protection by influencing interaction with NK cell receptors. The ability to assay a physical interaction between Ly-49 and MHC class I molecules should permit exploration of this hypothesis and determination of the significance of the C-type lectin homology of Ly-49 (9).

The current results clearly suggest that, in natural killing, MHC class I-associated target cell resistance may be attributed to specific recognition of target cell MHC class I mole-

cules by NK cell receptors such as Ly-49. When considered together with previous functional studies with Ly-49<sup>+</sup> effector cells, the physiologic role of physical interaction between Ly-49 and H-2D<sup>d</sup> is probably not cell adhesion per se even though we have used cell adhesion to demonstrate this interaction. The quantitative assay demonstrated that a lower level of Ly-49 expression, comparable to constitutive expression on Ly-49<sup>+</sup> NK cells was insufficient to produce detectable cell adhesion. This suggests that the binding affinity may be relatively low, i.e., TCR-MHC interaction (30), or alternatively, Ly-49 could bind with high affinity to only a subset of H-2D<sup>d</sup> molecules, perhaps those displaying specific peptides. Although further studies are necessary to delineate the precise mechanism by which D<sup>d</sup> engagement of Ly-49 subsequently influences NK cell cytotoxicity, we favor the hypothesis that physical engagement of Ly-49 subsequently triggers an inhibitory signal that overrides NK cell activation (6, 7, 11). This interpretation also suggests that an NK cell possesses another receptor responsible for NK cell activation in natural killing. Therefore, the susceptibility of a target to a particular NK cell may be determined by target expression of specific MHC class I molecules and a putative ligand for the activation receptor, as well as expression of the appropriate specific NK cell receptors.

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